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Optimization of the solvent extraction of phenolics and antioxidants from waste Cauliflower leaves (*Brassica oleracea* L.) using Response Surface Methodology (RSM)

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ABSTRACT:
This study investigates the extraction characteristics and optimal parameters for the solvent extraction of phenolic compounds and their antioxidants from waste cauliflower leaves (*Brassica oleracea* L.). Response surface methodology (RSM) by central composite design (CCD) was employed to statistically optimize the extraction conditions by varying three parameters: methanol concentration (X_1: 60-90%), extraction temperature (X_2: 55-100°C) and extraction time (X_3: 45-100min). The analysis of variance (ANOVA) indicated that the linear coefficients of methanol concentration(X_1) and extraction temperature(X_2) have significant effect on extraction of higher yield of phenolic compounds. The linear terms of methanol concentration(X_1), extraction temperature(X_2)and interaction between methanol concentration and extraction temperature(X_1X_2),methanol concentration and extraction time(X_1X_3), as well as extraction temperature and extraction time(X_2X_3) had significant effect on higher capability of antioxidant effects on the yield of phenolic compounds ($p<0.05$). The combined effects of independent variables were investigated and the optimal extraction conditions were obtained as methanol concentration (X_1): 75%, extraction temperature(X_2): 115.34°C and extraction time(X_3): 72.5 min. under this conditions to achieve the highest yield of total phenolic content: 485.58 mg GAE/100gm, total flavonoid content: 102.91mgQuer/100mg, and the corresponding antioxidant activities of%DPPHsc:84.77%, FRAP: 3484µgmol Fe(II)/gm and %H_2O_2sc: 80.85 from waste cauliflower leaves powder. The predicted optimum conditions were validated with independent sets of experimental data collected at the predicted optimum cooperating conditions. The results if this validation study showed good matching. Thus, this optimization procedure could be helpful in the food and pharmaceutical industry, especially for the extraction of high quality bioactive products from natural sources.

Keywords: *Brassica oleracea* L., Response surface methodology, Antioxidants, Phenolic compounds, Central composite design.

INTRODUCTION

Oxygen has a major role for our survival. Under certain circumstances it causes deleterious effect on human body. It occurs by the formation of reactive oxygen species (ROS). ROS is generated during aerobic metabolism [1, 2]. Oxygen ions, superoxide radicals, hydroxyl radicals are examples of ROS. The imbalance between the oxidant effect of ROS and antioxidant results in the onset of oxidative stress. Oxidative stress causes development in tissue damage and play major role by the initiation of carcinogenesis [3, 4, 5].ROS is overproduced by oxidative stress and results in peroxidation of lipid which ultimately causes lipid layer damage. [6,7] Antioxidant protects our body from such adverse effect by its defense mechanism. Synthetic antioxidants such as butylated hydroxyl toluene, butylated hydroxyl anisole are commonly used, but they have a carcinogenic and toxic effect[8,9]. Phenolic compounds, which are large group of secondary metabolites in higher plants, are responsible for antioxidant, anti-inflammatory, anti-cancerous and hepatoproductive and antibacterial activities [10, 11, 12].

Cauliflower is a tropical crop of India and China in the family Brassicaceae. Research focused on brassica vegetables has implied it has high content of secondary metabolites [13, 14]. It has been reported that the high intake of brassica vegetables reduces the chances of cardiovascular disease and other degenerative diseases [15]. Brassica vegetables are rich in phenolic compounds which is the major antioxidant of this plant [16]. Many parameters such as solvent, solvent concentration, extraction temperature, particle size and solid to liquid ratio can influence the extraction and isolation of desired product. The conventional approach for optimization of multivariate is done by using one variable at a time. Response surface methodology can solve simultaneously multivariate equations. RSM, which was introduced by Box and Wilson [17], proved to a useful software tool for solving multivariable system. RSM is a collection of mathematical and statistical techniques for empirical model building [18].The response can be represented graphically or contour plot that helps to visualize the response surface. RSM reduces the number of experiments and provides a mathematical model [19, 20]. RSM has been widely applied for optimization and isolation of valuable products from natural sources in the food and pharmaceutical industry [21, 22].

The objective of this study was to determine the optimum extraction conditions for waste cauliflower leaves in order to maximize the yield of total phenolic contents (TPC), total flavonoids content (TFC) and their highest capability of antioxidant activities by response surface methodology. All the data were analyzed by using Design Expert (version 8.0.7.1, Stat-Ease, Inc, Minneapolis, MN, USA) statistical software. In the current experiment five levels (-1.682,-1, 0, +1, +1.682) and three factors (TPC, TFC and the antioxidant activities) central composite design was employed to examine the optimum conditions.

MATERIALS AND METHODS

Plant material and sample preparation

Cauliflower leaves were obtained from a local vegetable market in Kolkata, India. It was washed several times with tap water, dried in shade for 1week and the whole leaves were ground to a fine powder using kitchen blender (Bajaj Electronics, Ltd). The powder was passed through 60mesh size screen and kept in airtight desiccator for further extraction experiments.

Chemicals and reagents

2, 4, 6-tripyridyl-s-triazine (TPTZ), Folin-Ciocalteu's phenol reagent (FCR) and Gallic acid of Himedia laboratories Pvt. Ltd. Mumbai, India; 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Quercetin and Rutin of Sigma–Aldrich, MO, USA; Aluminium chloride, Sodium carbonate, Sodium hydroxide, Ferric chloride, Hydrogen peroxide and analytical grade solvents of Merck, Mumbai, India were used in the study.

Extraction of phenolic compounds and antioxidants

Extraction of phenolic compounds and antioxidants from Cauliflower leaves were carried out by using methanol as a solvent based on the *Aybastier, O* et.al.method[23]. The extraction was carried out by using 1g of powdered sample of Cauliflower leaves. The sample was transferred to a 100mL conical flask containing 30mL miscible liquid-liquid mixture of methanol and water. The cotton plugged conical flask was placed on a constant temperature water bath equipped with shaking arrangement. Sets of experiments were carried out by varying methanol concentration (60-90%) in methanol-water mixture, temperature (55-100˚C) and time (45-100 minutes). Values of operating parameters were set according to the CCD Table. Each extract, obtained by filtration of content of conical flask through Whatman No: 1 filter paper was analyzed for TPC, TFC and antioxidant activities.

Total phenolic content assay (TPC)

Total phenolic content of the extract was estimated spectrophotometrically (Varian Cary 50 UV-Spec) using Folin-Ciocalteu's reagent (FCR) according to the method described by *Singleton et.al.* with slight modifications[23]. Approximately 0.3mLof each extract solution was mixed with 1.8mL of Folin-Ciocalteu reagent and allowed to stand at room temperature for 5 min. After 5 min, 1mL of 20 %(w/v) aqueous sodium carbonate were added, the volume was made up to 5mL with distilled water. The blank contained all the reaction reagents except the extract. After 2h of incubation at ≈25°C, the absorbance was measured at 760nm by using spectrophotometer and gallic acid was used as a standard. The results were expressed as mg gallic acid equivalents GAE/g sample.

Total Flavonoids Content assay (TFC)

Aluminium chloride spectrophotometric method was used to determine the total flavonoid content according to the method described by Gan C. Y et al. [24]. Approximately 0.5mL of each extract solution mixed with 1.5mL of 10% aluminium chloride, 0.1mL of 1M potassium acetate and 2.9mL of distilled water. The blank contained all the reaction reagents except the extract. It is allowed to stand at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415nm by using spectrophotometer. The total flavonoid content was expressed as quercetin equivalents QUE/g extract.

Determination of antioxidant capacity

%DPPH scavenging assay

The antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability, using the Table radical DPPH*, according to the method suggested by *Ramadan et al.* [25]with slight modifications. Aliquot of each extract (0.1mL) was added to 3mL of ethanolic solution of DPPH (0.1µM). The mixture was shaken vigorously and allowed to stand for 30 minutes in the dark, and the absorbance was measured at 517nm against a blank. The capability to scavenge the free radical DPPH in percentage of sample (%$DPPH_{SC}$) was calculated using the formula;

$$\%DPPH_{SC} = (A_0 - A_1) \times 100/A_0 \quad (1)$$

whereA_0= absorbance of the control; A_1 = absorbance of the sample.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay is based on the ability of extract to reduce Fe^{3+} in 2, 4, 6-tripyridyl -s-triazine (TPTZ) solution to Fe^{2+} and create blue colored complex Fe^{2+}-TPTZ. The FRAP assay was used to estimate the antioxidant potential of the extract, according to *Benzie* and *Strain* method [26]. The FRAP reagent was prepared using 300mM acetate buffer (3.1g Sodium acetate, and 16mL Acetic acid) at pH 3.6, 10mM TPTZ (2,4,6-tripyridyl -s-triazine) solution in 40mM hydrochloric acid solution, and 20mM $FeCl_3.6H_2O$ solution in distilled water. The acetate buffer (25mL) and TPTZ (2.5mL) were mixed together with $FeCl_3.6H_2O$ (2.5mL). The temperature of the solution was adjusted to 37˚C before it was used. Plant extract (40µL) were allowed to react with the FRAP solution (3mL) for 30min under dark conditions. The

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absorbance was measured at 593nm.The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results were expressed in µM Fe (II)/g dry mass and compared with ascorbic acid as a standard

% H_2O_2 scavenging assay

H_2O_2 scavenging ability of extracts was carried out by *Nabavi et al*. method with slight modifications [27]. H_2O_2 solution (40 mM) was prepared in phosphate buffer (pH 7.4). The each extract of waste cauliflower leaves (1mL) in phosphate buffer (3.4 mL) was added to H_2O_2 solution (0.6 mL; 40 mM). The absorbance of the reaction mixture was recorded at 230 nm. Blank solution contained the phosphate buffer without H_2O_2.

The percentage of H_2O_2 scavenging of each extract was calculated using the following formula

$$\%\ H_2O_2sc = (A_0 - A_1) \times 100/A_0 \qquad (2)$$

whereA_0 = absorbance of the control; A_1 = absorbance of the sample

Experimental design

Response surface methodology (RSM) using central composite design (CCD)was applied to identify optimum levels of three variables, namely, methanol concentration(X_1),extraction temperature(X_2) and extraction time(X_3) on the highest yield of total phenolic (TPC) and flavonoid (TFC) contents and shows their maximum antioxidant activities for waste cauliflower leaves. Design Expert (version 8.0.7.1, Stat-Ease, Inc, Minneapolis, MN, USA) statistical software was used. For data analysis and model establishing the software, five levels three factor experimental design with six replicates at the centre point was adopted. The three factors (TPC, TFC and antioxidant activities), five levels (-1.682,-1,0,+1,+1.682) as coded on independent variables and six replicates as shown in Table 1. The central composite design comprised of 20 experimental runs with 8 factorial points, 6 axial points at a distance of ± 1.682 from the central points as shown in Table 2.

Table 1: Experimental range of coded and actual values for central composite design (CCD)

Coded variables (X_j)	Symbol	-1.682	-1	0	+1	+1.682
MeOH (%)	X_1	49.77	60	75	90	100.23
Ext. Temp(°C)	X_2	39.66	55	77.5	100	115.34
Ext. Time (min)	X_3	26.25	45	72.5	100	118.75

The experimental data were fitted by a second order polynomial equation in order to correlate the dependent variable to the independent variable. The generalized second order polynomial equation to obtain coefficients of the equation as follows

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j + \varepsilon \qquad (3)$$

Where Y is response (TPC, TFC ,%DPPHsc, FRAP and $\%H_2O_{2sc}$), βo is the model contant, βi, βii and βij are the model coefficients, Xi and Xj are coded value of the independent variables, and ε is the error. The additional confirmations of the experiments were subsequently conducted to verify the statistical experimental analysis.

Thin layer chromatographic analysis of Phenolic compounds

Thin layer chromatography of the optimized extract was run in the mobile phase solvent (methanol - benzene - water, 60:35:5, v/ v/ v) at room temperature of ≈25°C. The TLC plate was prepared by using the glass plates (100×200mm) coated with silica gel GF_{254} (0.2-0.3mm) paste were dried naturally (atmospheric), subsequently they were activated at 100 ˚C for 30 minutes and were cooled at room temperature (≈ 25ºC). The optimized extract was concentrated and were spotted on the TLC plate and the diameter of the spot in each chromatogram was normally about 5mm and 2 authentic markers of flavonol (quercetin) and flavonoid glycoside (rutin) obtained commercially were co-chromatographed. Identification of the flavonoids in the extracts was identified under UV light after the application of ammonia [28].

Statistical data analysis

The experimental result of the response surface methodology was analyzed using Design Expert (version 8.0.7.1, Stat-Ease, Inc, Minneapolis, MN, USA) statistical software. All the results were expressed as the mean ± standard deviation. The optimal extraction conditions were analyzed by one way analysis of variance (ANOVA), the three dimensional (3D) response surfaces and contour plots. The software generated regression coefficient for each of the independent variables and the significance was determined using the p value generated through t-test.

RESULTS AND DISCUSSION

The effect of three parameters namely, methanol concentration(X_1), extraction temperature(X_2) and extraction time(X_3) were analyzed. The parameters which gave highest yield TPC, TFC and their antioxidant activity were selected as the optimum parameter. The five responses were TPC, TFC, %DPPHsc, FRAP and $\%H_2O_2sc$. Response surface methodology (RSM) using central composite design (CCD) was applied which resulted in 20 experiments including six replicates, were predicted and experimental value are shown in Table 2. Among the 20 experiments, it was observed that the yield of TPC and TFC contents ranged from 100.75-485.58 mg GAE/100gm and 19.13-102.91 mg Quer/100gm respectively. The range of three antioxidant activities of %DPPHsc: 10.97-84.77, FRAP: 1213-3938µgmol Fe(II)/gm and $\%H_2O_2sc$: 13.91-80.85 were recorded under the experimental conditions. The highest level of TPC (485.58 mg GAE/100gm)and TFC (102.91 mg

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Quer/100gm) and shows their maximum antioxidant activities are (%DPPHsc:84.77, %H_2O_2sc: 80.85) were obtained with 75% methanol at 115.34°C for 72.5 min. The maximum FRAP: 3938 µgmol Fe (II)/gm was observed 75% methanol at 39.65°C for 72.5 min. The maximum points are obtained under the same condition. Therefore an optimal conditions for the extraction of TPC, TFC and antioxidants were X_1=75%, X_2=115.34°C and X_3=72.5min.

Table 2: Central Composite Design with experimental responses and predicted responses

Run	Std. order	Coded Variable levels			Experimental value (Y1)					Predicted Value (Y2)				
		MeOH % (X_1)	Ext. Temp °C (X_2)	Ext. Time Min (X_3)	TPC (mg GAE /100gm)	TFC (mg Quer /100gm)	%$DPPH_{sc}$	FRAP (µg mol Fe(II) /gm)	%H_2O_{2sc}	TPC (mg GAE /100gm)	TFC (mg Quer /100gm)	%$DPPH_{sc}$	FRAP(µg mol Fe(II)/gm)	%H_2O_{2sc}
1	18	75	77.5	72.5	200.34	35.51	17.72	3138	38.96	213.88	46.41	26.14	3025	37.88
2	9	49.77	77.5	72.5	363.24	98.48	32.74	3122	34.69	258.89	67.89	31	3404	36.23
3	7	60	100	100	229.29	69.97	29.1	2298	62.67	322.29	65.73	34.93	2574	56.17
4	4	90	100	45	383.78	46.99	13.47	1213	38.96	294.68	49.79	39.58	1892	49.47
5	6	90	55	100	127.02	30.71	15.56	3361	38.96	79.55	17.48	6	3506	29.1
6	5	60	55	100	108.15	32.6	31.65	3454	15.71	133.07	43.03	12.7	3365	26.28
7	13	75	77.5	26.25	100.75	47.67	80.33	3582	18.75	255.58	59.83	35.68	2727	29.87
8	8	90	100	100	155.75	30.71	35.25	3185	62.21	268.87	40.19	28.23	3360	59
9	14	75	77.5	118.74	233.24	32.89	13.32	3377	38.96	192.18	38.33	16.06	3322	45.88
10	10	100.22	77.5	72.5	158.56	19.13	15.54	2322	19.05	168.88	24.93	20.59	2530	40.18
11	1	60	55	45	117.47	45.48	11.64	3711	16.9	158.88	52.63	24.05	4126	16.76
12	16	75	77.5	72.5	196.63	33.48	18.16	3119	34.82	213.88	46.41	26.14	3025	37.88
13	17	75	77.5	72.5	200.15	33.07	13.83	3112	36.96	213.88	46.41	26.14	3025	37.88
14	15	75	77.5	72.5	199.93	35.24	14.71	3169	38.96	213.88	46.41	26.14	3025	37.88
15	11	75	39.65	72.5	102.81	34.65	10.97	3938	13.91	54.63	27.31	7.44	3487	12.73
16	19	75	77.5	72.5	201.44	53.8	16.81	3180	38.96	213.88	46.41	26.14	3025	37.88
17	2	90	55	45	142.57	55.45	21.27	1887	46.69	105.36	27.09	17.35	2201	19.58
18	3	60	100	45	375.44	56.79	29.97	2728	45.98	348.2	75.33	46.28	3173	46.65
19	20	75	77.5	72.5	195.48	32.74	16.07	3122	34.69	213.88	46.41	26.14	3025	37.88
20	12	75	115.34	72.5	485.58	102.91	84.77	3484	80.85	373.08	65.5	44.83	2562	63.02

Model fittings

The significance of the quadratic model is analyzed by ANOVA shown in Table 3. The F-test and the p-value analyses the significance of each coefficient. Corresponding variables are more significant when the F-value is greater and p-value is smaller. A p-value less than 0.05 indicated that the coefficient was statistically significant. The F-value (6.27) and p-value (0.0276) also implied that the model was significant. The model was judged by the determination of multiple regression coefficients (r^2) and lack of fit was also significant. The fitted models for TPC, TFC, %$DPPH_{SC}$, FRAP, H_2O_2 are given in eqn. (4) - (8).

$$TPC= +213.88 -26.76X_1+94.66X_2 -12.90X_3 \quad (4)$$

$$TFC= +46.41 -12.77X_1 +11.35X_2 -4.80X_3 \quad (5)$$

$$\%DPPHsc=+26.14 -3.35X_1 +11.11X_2 -5.67X_3 \quad (6)$$

$$FRAP=+3025.10-284.87X_1-274.77X_2 +176.78X_3+161.13X_1X_2+516.63X_1X_3+40.63X_2X_3 \quad (7)$$

$$\%H2O2sc=+37.88+1.41X_1+14.95X_2+4.76X_3 \quad (8)$$

Analysis of the model

TPC

Table 3 shows that solvent concentration(X_1) and extraction temperature(X_2) significantly ($p<0.05$) affects the yield of TPC. The correlation coefficient of determination (r^2) for TPC was 0.6174.The p-value for the lack of fit was highly significant (<0.0001).Figure1a and 1b shows the 3D response surfaces and contour plot shows that the yield of TPC (485.58 mg GAE/100gm) depends on the methanol concentration and extraction temperature. Eqn-(4) shows the relationship between TPC yield and extraction parameters.

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Table 3: Analysis of Variance (ANOVA) for the quadratic polynomial mode

Source	Sum of Squares	df[a]	Mean Square	F Value[b]	p value[c]
TPC(mg GAE/100gm)[d]					
Model	1.34E+05	3	44810.78	8.61	0.0012
A-Acetone conc	9779.74	1	9779.74	1.88	0.1895
B-Extraction temp	1.22E+05	1	1.22E+05	23.5	0.0002
C-Extraction time	2274.08	1	2274.08	0.44	0.5181
Residual	83312.04	16	5207		
Lack of Fit	83284.1	11	7571.28	1354.74	< 0.0001
Pure Error	27.94	5	5.59		
Cor Total	2.18E+05	19			
TFC(mg Quer/100gm)[e]					
Model	4302.4	3	1434.13	4.66	0.016
A-Acetone conc	2227.89	1	2227.89	7.23	0.0161
B-Extraction temp	1759.63	1	1759.63	5.71	0.0295
C-Extraction time	314.88	1	314.88	1.02	0.327
Residual	4927.65	16	307.98		
Lack of Fit	4594.67	11	417.7	6.27	0.0276
Pure Error	332.98	5	66.6		
Cor Total	9230.05	19			
%$DPPH_{sc}$[f]					
Model	2279.82	3	759.94	2.06	0.1455
A-Acetone conc	153.17	1	153.17	0.42	0.5281
B-Extraction temp	1687	1	1687	4.58	0.0481
C-Extraction time	439.65	1	439.65	1.19	0.2908
Residual	5893.04	16	368.32		
Lack of Fit	5878.66	11	534.42	185.87	< 0.0001
Pure Error	14.38	5	2.88		
Cor Total	8172.86	19			
FRAP(µgmol Fe(II)/gm)[g]					
Model	4.92E+06	6	8.20E+05	3.41	0.0303
A-Acetone conc	1.11E+06	1	1.11E+06	4.6	0.0514
B-Extraction temp	1.03E+06	1	1.03E+06	4.28	0.059
C-Extraction time	4.27E+05	1	4.27E+05	1.77	0.206
AB	2.08E+05	1	2.08E+05	0.86	0.37
AC	2.14E+06	1	2.14E+06	8.86	0.0107
BC	13203.13	1	13203.13	0.055	0.8185
Residual	3.13E+06	13	2.41E+05		
Lack of Fit	3.13E+06	8	3.91E+05	489.39	< 0.0001
Pure Error	3994	5		798.8	
Cor Total	8.05E+06	19			
%$H2O2_{sc}$[h]					
Model	3388.02877	3	1129.342923	8.7189948	0.0012
A-Acetone conc	27.15287166	1	27.15287166	0.20963141	0.6532
B-Extraction temp	3051.421443	1	3051.421443	23.5582365	0.0002
C-Extraction time	309.4544547	1	309.4544547	2.38911647	0.1417
Residual	2072.42775	16	129.5267344		
Lack of Fit	2051.1166	11	186.4651455	43.7482598	0.0003
Pure Error	21.31115	5	4.26223		
Cor Total	5460.45652	19			

[a] Degrees of freedom
[b] Test for comparing model variance with residual (error) variance
[c] Probability of seeing observed f-value if the null hypothesis
[d] The coefficient of determination (r^2) of the model was 0.6174
[e] The coefficient of determination (r^2) of the model was 0.4661
[f] The coefficient of determination (r^2) of the model was 0.2790
[g] The coefficient of determination (r^2) of the model was 0.3186
[h] The coefficient of determination (r^2) of the model was 0.6205

TFC

Table 3 shows that solvent concentration(X_1) and extraction temperature(X_2) significantly ($p<0.05$) effects the yield of TFC. The correlation coefficient of determination (r^2) for TFC was 0.4661. The p-value for the lack of fit has also been highly significant (0.0295). Figure 2a and 2b shows the 3D response surfaces and contour plot of TFC represents it as a function of

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methanol concentration(X_1) and extraction temperature(X_2). Thus the model Eqn–(5) showing the relationship between TFC yield and extraction parameters is valid.

Antioxidant activities (%DPPHsc, FRAP, %H2O2sc)

Table 3 shows that the extraction temperature has a significant ($p<0.05$) effect on %DPPHsc. The p-value for the lack of fit was also significant (<.0001) and the correlation coefficient of determination (r^2) of the model was 0.2790 which indicates it is a good fit for the model. Figure 3a and 3b shows the 3D responses and contour plot which represents it is a function of both methanol concentration(X_1) and extraction temperature(X_2).Accordingly, the model Eqn–(6) showing the relationship between %DPPHsc activity and extraction parameters is valid.

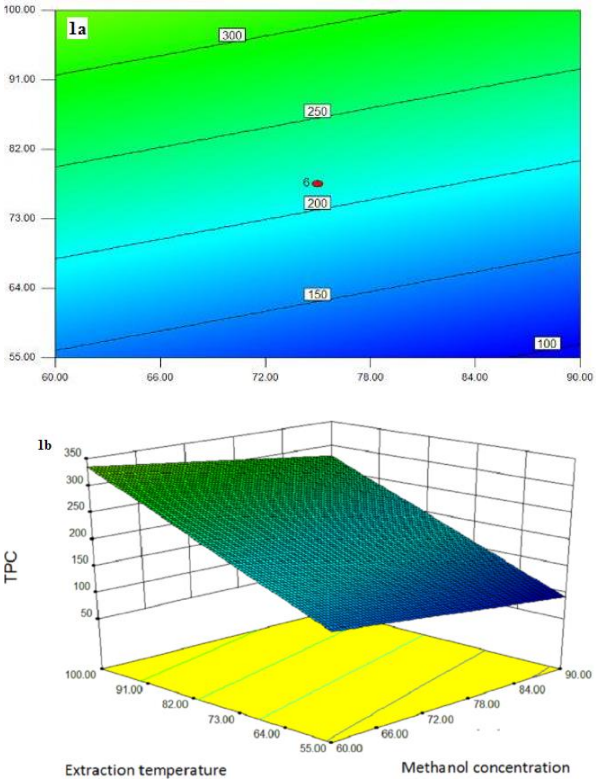
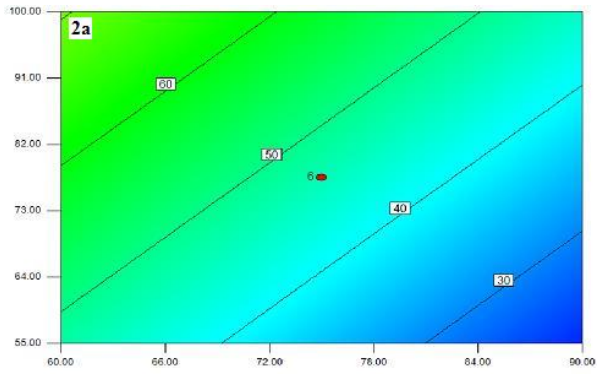


Figure 1a and 1b 3D response surface and contour plot of the combined effects of methanol concentration and extraction temperature on highest yield of TPC when extraction time was held at fixed level.



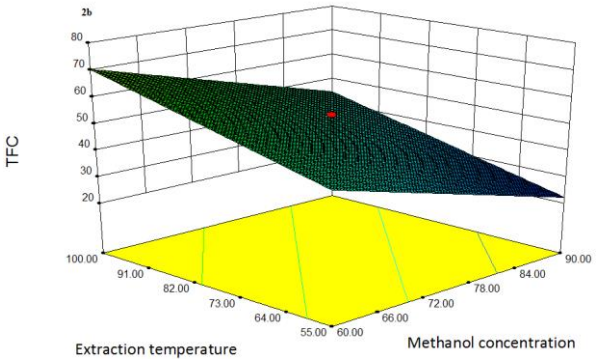


Figure 2a and 2b 3D response surface and contour plot of the combined effects of methanol concentration and extraction temperature on highest yield of TFC when extraction time was held at fixed level.

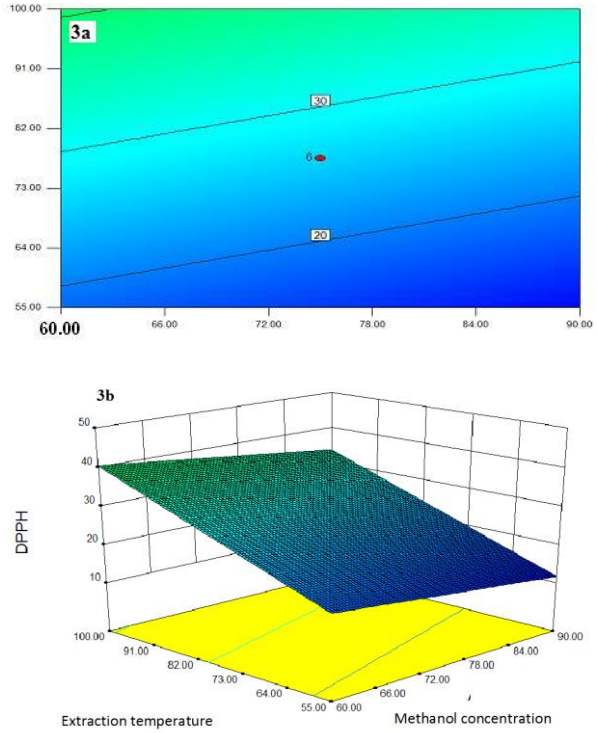
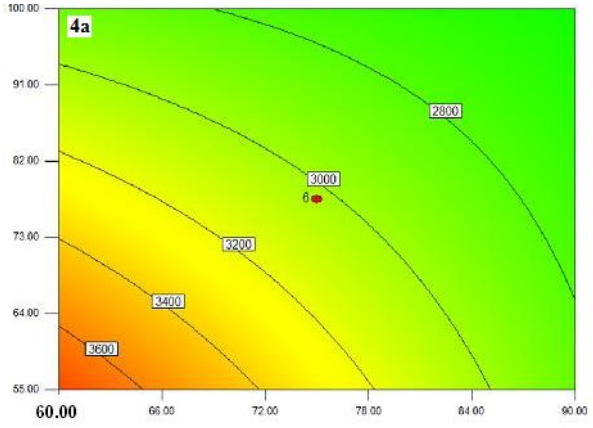


Figure 3a and 3b 3D response surface and contour plot of the combined effects of methanol concentration and extraction temperature on highest antioxidant activities of %DPPHsc assay when extraction time was held at fixed level



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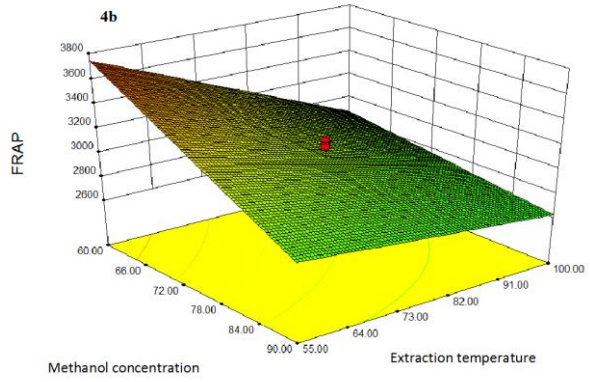
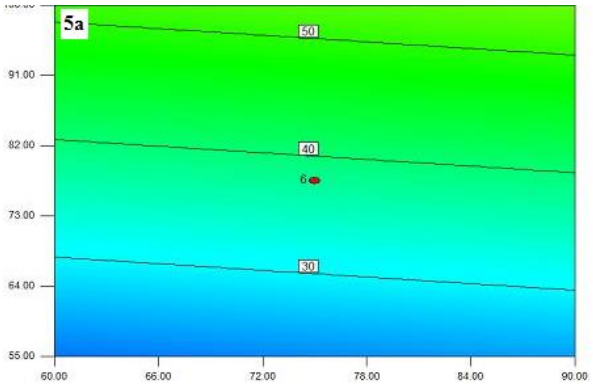


Figure 4a and 4b 3D response surface and contour plot of the combined effects of methanol concentration and extraction temperature on highest reducing power FRAP assay when extraction time was held at fixed level.



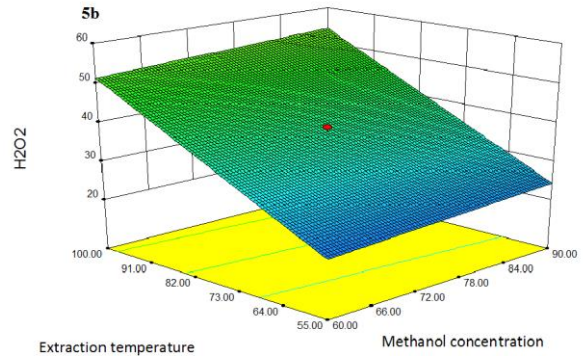


Figure 5a and 5b 3D response surface and contour plot of the combined effects of methanol concentration and extraction temperature on highest antioxidant activities of $\%H_2O_{2sc}$ assay when extraction time was held at fixed level.

From the Table 3 the effects on antioxidants power of FRAP was shown in significantly ($p<0.005$) effected by the interaction of methanol concentration and extraction time. The p-value for lack of fit is also significant ($p<0.0001$). The coefficient of determination (r^2) of the model was 0.3186. Figure 4a and 4b shows the 3D response and contour plot which represents it is a function of methanol concentration(X1) and extraction temperature (X2).Model Eqn-(7) shows relation between result of FRAP and extraction parameters are valid.

The result of $\%H_2O_{2sc}$ obtained from Table 3, the values are significant ($p<0.005$) for extraction temperature. p-value is also significant for lack of fit. The correlation coefficient of determination (r^2) of the model was 0.6205. 3D responses and contour plot shows it is a function of both extraction temperature and methanol concentration. Figure 5a and 5b shows the 3D response and contour plot which represents it as a function of methanol concentration(X_1) and extraction temperature(X_2). Model Eqn-8 shows that the relation between the value of $\%H_2O_{2sc}$assay and extraction parameters are valid.

Verification of the model

The suitability of verification experiment was performed to find the reliability optimization result.Table 4 shows the verification experiment under optimum conditions based on each individual response with predicted and experimental values. The verification experiment was conducted under optimum conditions based on combination of responses and small deviation was observed as compared to predicted values. The optimum operation conditions for TPC, TFC, %DPPHsc, FRAP and $\%H_2O_{2sc}$was observed at methanol concentration 75% ,extraction temperature 115.34°C ,extraction time 72.5min.The maximum value obtained from TPC, TFC, %DPPHsc, FRAP and $\%H_2O_{2sc}$ were:485.58(mg GAE/100gm), 102.91 mg (Quer/100gm), 84.77%, 3484µgmol Fe (II)/gm, 80.85% respectively. This model implied that there was a good fit between the experimental value and those predicted by the regression model.

Table 4: Verification of individual experimental data and predicted values under optimum conditions.

Dependent variables	Predicted value	Experimental value
TPC (mg GAE/100gm)	373.08	485.58
TFC (mg Quer/100gm)	65.5	102.91
$\%DPPH_{sc}$	44.83	84.77
FRAP (µgmol Fe(II)/gm)	2562	3484
$\%H_2O_{2sc}$	63.02	80.85

Thin layer chromatographic analysis of phenolic compounds

Figure 6 shows the presence of flavonoid glycosides, flavonols and phenolic acids in the optimized extracts.

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Figure 6 Thin layer chromatographic profiles of phenolic contents.

CONCLUSIONS

Application of RSM with statistical analysis based on central composite design enabled to optimize solvent extraction of phenolic compounds and their enhanced antioxidant activities from waste cauliflower leaves was successfully employed. The optimal conditions were 75% methanol at temperature 115.34 ˚C in 72.5 min under this conditions for obtaining greater TPC(485.58mg equivalent GAE/100gm), TFC(102.91 mg equivalent Quer/100gm) and antioxidant activities(%DPPHsc: 84.77%, FRAP: 3484µgmol Fe (II)/gm and $\%H_2O_2$sc:80.85%) . All the experimental results well matched with predicted results. Based on 3D response surfaces and contour plots, methanol concentration(X_1) and extraction temperature(X_2) were the most important factors on the yield of TPC, TFC and shows their maximum antioxidant activities. Accordingly, the procedure may be helpful in the food and pharmaceutical industry in studying the extraction of high quality bioactive products from natural sources.

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